Effects of Receptor Binding Specificity of Avian Influenza Virus on the Human Innate Immune Response[∇][†]

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Humans infected by the highly pathogenic H5N1 avian influenza viruses (HPAIV) present unusually high concentrations in serum of proinflammatory cytokines and chemokines, which are believed to contribute to the high pathogenicity of these viruses. The hemagglutinins (HAs) of avian influenza viruses preferentially bind to sialic acids attached through α2,3 linkages (SAα2,3) to the terminal galactose of carbohydrates on the host cell surface, while the HAs from human strains bind to α2,6-linked SA (SAα2,6). To evaluate the role of the viral receptor specificity in promoting innate immune responses in humans, we generated recombinant influenza viruses, one bearing the HA and neuraminidase (NA) genes from the A/Vietnam/1203/2004 H5N1 HPAIV in an influenza A/Puerto Rico/8/1934 (A/PR/8/34) backbone with specificity for SAα2,3 and the other a mutant virus (with Q226L and G228S in the HA) with preferential receptor specificity for SAα2,6. Viruses with preferential affinity for SAα2,3 induced higher levels of proinflammatory cytokines and interferon (IFN)-inducible genes in primary human dendritic cells (DCs) than viruses with SAα2,6 binding specificity, and these differences were independent of viral replication, as shown by infections with UV-inactivated viruses. Moreover, human primary macrophages and respiratory epithelial cells showed higher expression of proinflammatory genes after infection with the virus with SAα2,3 affinity than after infection with the virus with SAα2,6 affinity. These data indicate that binding to SAα2,3 by H5N1 HPAIV may be sensed by human cells differently than binding to SAα2,6, inducing an exacerbated innate proinflammatory response in infected individuals.

Influenza A viruses, due to their mode of transmission and the high mutation frequency of their genomes, are among the leading pandemic disease threats. From 1997 until today, highly pathogenic avian influenza viruses (HPAIV) of subtype H5N1 have caused several outbreaks in birds that have resulted in a high mortality rate and that have been accompanied by occasional transmission to humans. Infections in humans often result in a severe and rapidly progressive pneumonia and subsequent systemic disease, with a fatal outcome in approximately 60% of the total cases reported to the Word Health Organization to August 2010 (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_08_31/en/index.html). Humans infected by H5N1 HPAIV present unusually high serum concentrations of chemokines and proinflammatory cytokines, and it is thought that this cytokine dysregulation may contribute to disease severity (5, 7, 11, 25, 32). Furthermore, elevated expression of MxA and alpha interferon (IFN-α) has been observed in autopsy lung tissue from an H5N1 virus-infected patient (46).

Avian strains of influenza virus are not efficient at infecting humans (4), and direct transmission from human to human has been reported only in close family clusters, with very limited

spread of the virus (54). There are some receptor restrictions for avian influenza viruses in human airways that may account for the poor ability of avian strains to establish infections in humans (22, 29–31, 49). The capacity of the influenza viruses to infect birds or humans seems to be defined in part by the binding specificity of the hemagglutinin (HA), the major glycoprotein on the influenza virus surface. Generally, HAs of human strains of influenza virus preferentially bind sialic acids attached through an α2,6 linkage to the terminal galactose (SAα2,6) of the oligosaccharides on the cell surface. These types of linkages are frequent in human respiratory epithelia (36). In contrast, the HA of avian strains bind preferentially to α2,3-linked sialic acids (SAα2,3), which are abundant in the avian intestinal tract (33).

Interaction of the HA with sialylated glycans on the cell surface is necessary for the infection of host cells and the transmission and virulence of influenza viruses (22, 37). Mutations that alter the receptor binding specificity of avian viruses could be important for the crossover of the virus from avian to human hosts, as well as for allowing direct human-to-human transmission (29). Several amino acid changes in the HA receptor binding site of avian viruses have been shown to change the receptor specificity from SAα2,3 to SAα2,6 (8, 43, 53). Recently, it has been reported that the A/Indonesia/5/2005 H5N1 HPAIV, which bears point mutations that switch the receptor preference to SAα2,6, shows strong attachment to human tissue sections from different regions of the respiratory tract; in contrast, binding of the virus with wild-type (WT) HA is minimal and restricted to tissue sections from the lower respiratory tract (8). These findings suggest that alterations in the receptor binding specificity could make HPAIV capable of

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infecting human hosts. Examination of the receptor specificity of different human and avian H2 viruses and of human, avian, and equine H3 influenza viruses by Connor et al. (9) revealed a correlation between the receptor specificities of these viruses and the residues at positions 226 and 228 of the HA. Specifically, they observed that viruses binding terminal SAα2,6 had residues L and S at these positions but that viruses binding SAα2,3 presented Q and G, respectively. Also, they observed that amino acids L and S were conserved at positions 226 and 228 in the human isolates but that Q and G were frequently found in avian and equine isolates. Later, using glycan arrays, it was shown that the change at positions 226 and 228 to L and S, respectively, in the HA of the H5N1 A/Vietnam/1203/2004 virus altered its receptor specificity, permitting binding to a natural human SAα2,6 glycan (43, 53).

Dendritic cells (DCs) have an essential role in initiating the innate immune response. This cell type presents an important number of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) in different cell locations. Hence, pathogens can be "sensed" by cytosolic receptors, like retinoic acid inducible gene I (RIG-I)-like helicases (RLH) (19), in endocytic compartments by several of the Toll-like receptors (TLRs) (21) or on the cell surface by other TLRs and c-type lectin receptors (CLRs) (20, 48). Besides having this function of pathogen recognition, these receptors induce the activation of different signaling cascades, leading to the modulation of gene expression. Moreover, DCs are also professional antigen-presenting cells, forming the main link between innate and adaptive responses (2, 40).

In this work, we hypothesized that the receptor specificity of the avian influenza virus may be related to exacerbated levels of proinflammatory cytokines and chemokines in infected humans. As a strategy to tackle this question, we generated recombinant influenza viruses with different receptor specificities by introducing the mutations Q226L and G228S into the HA of the A/Vietnam/1203/2004 virus. Then, following characterization of the receptor binding specificities of the viruses bearing 226Q 228G (wild-type genotype) and 226L 228S (mutant genotype) by solid-phase and flow cytometry binding assays, we studied the expression profiles of proinflammatory genes and proteins in primary human DCs and subsequently in macrophages and human tracheobronchial epithelial (HTBE) cells upon infection with those viruses. The virus encoding WT HA, which showed SAα2,3-preferential binding, induced higher levels of cytokines and chemokines in DCs, macrophages, and HTBE cells than the SAα2,6-binding mutant. Our data suggest an important role for receptor binding specificity in the activation of the innate immune response and offer a possible explanation for the hypercytokinemia developed in humans infected by HPAIV.

MATERIALS AND METHODS

Cells and viruses. Human primary dendritic cells and macrophages were generated from CD14$^+$ cells isolated from buffy coats of healthy human donors (New York Blood Center). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation (Histopaque; Sigma Aldrich) and incubated with anti-human CD14 antibody-labeled magnetic beads, and CD14$^+$ cells were purified using iron-based MiniMACS liquid separation columns (Miltenyi Biotech). For the generation of immature dendritic cells, CD14$^+$ cells were incubated at 37°C for 5 days at a concentration of 10^6 cells/ml in RPMI medium containing 10% fetal bovine serum (FBS) (HyClone; Thermo Scien-

tific), 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin–100 µg/ml streptomycin (Gibco, Invitrogen) (complete DC medium) and supplemented with 500 U/ml human granulocyte-macrophage colony-stimulating factor (hGM-CSF) and 1,000 U/ml human interleukin 4 (hIL-4) (Peprotech).

For macrophage generation, CD14$^+$ cells were incubated at 37°C for 10 days at a concentration of 0.5 × 10^6 cells/ml in complete DC medium and supplemented with 1,000 U/ml hGM-CSF, with fresh hGM-CSF added every 2 or 3 days.

Human tracheobronchial epithelial cells (Clonetics, Lonza) were grown in bronchial epithelial cell growth medium (BEGM), prepared by adding BEGM from a SingleQuot kit (Clonetics, Lonza) to 500 ml of bronchial epithelial cell basal medium (BEBM; Clonetics, Lonza). For differentiation, the cells were seeded on 12-mm Transwell filters (pore size, 0.4 µm; Corning) coated with collagen type I from human placenta (Sigma Aldrich) in 12-well plates (Corning) and were incubated with a 1:1 mixture of BEGM and Dulbecco's modified Eagle's medium (DMEM) (supplemented also with BEGM from a SingleQuot kit). When the cultures were confluent, liquid from the upper compartment was removed and cells were cultured in an air-liquid interphase for 4 to 6 weeks. Medium in the basal compartment was supplemented with 5 × 10^{-8} M retinoic acid (Sigma Aldrich). Total cell differentiation was assessed by β-tubulin surface staining.

MDCK and A549 cells were grown in minimal essential medium (MEM) and DMEM culture (Gibco, Invitrogen), respectively, supplemented with 10% FBS and penicillin-streptomycin (Gibco, Invitrogen).

Recombinant viruses. Recombinant influenza viruses were generated using reverse-genetics techniques as previously described (13). Viruses encoding HA with the WT sequence (Viet WT) and with the mutant sequence (Viet Mut) were constructed with the HALo WT or HALo Q226L G228S segment (HALo denotes an HA segment modified by the removal of the encoded polybasic cleavage site), neuraminidase (NA) from the H5N1 A/Vietnam/1203/2004 virus, and the six segments PB2, PB1, PA, NP, M, and NS from influenza A/Puerto Rico/8/1934 (A/PR/8/34) virus as previously described (39). BB was constructed using the HA and NA segments from the seasonal H1N1 A/Brisbane/59/2007 virus (kind gift from Adolfo Garcia-Sastre), and the rest of the segments were from A/PR/8/34 virus. All viruses were grown in 9-day-old embryonated chicken eggs (SPAFAS; Charles River Laboratories). All influenza viruses were titrated by plaque assay on MDCK cells by following standard procedures.

For the solid-phase binding assay, the viruses were partially purified through a 20% sucrose cushion according to standard procedures.

Flow cytometry-based binding assay. To carry out the analysis of the binding specificity of the influenza viruses, MDCK cells were infected at a multiplicity of infection (MOI) of 5 for 24 h with the corresponding influenza viruses. Next, cells were harvested, washed 3 times with cold phosphate-buffered saline (PBS), and then incubated with 10 µg/ml of the biotinylated glycans Neu5Acα2,3Galβ1,4GlcNAc-PAA (3′ SLN-PAA) and Neu5Acα2,6Galβ1,4GlcNAc-PAA (6′ SLN-PAA), provided by the Consortium of Functional Glycomics, and anti-M2 antibody E10 (Mount Sinai Hybridoma Shared Research Facility) for 2 h at 4°C. Then cells were washed with PBS-1% bovine serum albumin (BSA) and incubated with streptavidin-fluorescein isothiocyanate (FITC; Jackson Immunoresearch) and secondary anti-mouse rhodamine antibody (Jackson Immunoresearch). Both incubations were performed in the presence of 1 µM GS4071 (a kind gift of Christopher Basler) in order to avoid cleavage of the sialic acids of the synthetic polymers (18). Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson) and analyzed with FlowJo software.

Solid-phase binding assay. We also used a solid-phase binding assay to study the receptor specificity of the recombinant viruses as previously described by Matrosovich et al. (27), with some modifications. Briefly, 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with the specific purified influenza viruses at 20 µg/ml and incubated overnight at 4°C. Next, plates were blocked with Carbo-Free blocking solution (Vector Laboratories) for 30 min at room temperature (RT) and washed with washing buffer (0.1% BSA, 0.05% Tween 20, PBS), and the biotinylated glycan 3′ SLN-PAA or 6′ SLN-PAA was added at different concentrations; plates were then incubated for 2 h at RT. Next, samples were washed with PBS and incubated with streptavidin-horseradish peroxidase (HRP; R&D Systems) for 1 h at RT. Both incubations were performed in the presence of 1 µM GS4071. The HRP was developed with the substrate o-phenylenediamine (OPD; Invitrogen), the reaction was stopped with 1% sodium dodecyl sulfate (SDS), and the absorbance at 450 nm was analyzed in a microplate reader (BioTek).

Growth curves of recombinant viruses in cell lines. To examine viral replication, confluent MDCK and A549 cells were infected at multiplicities of infection (MOIs) of 0.001 and 0.1, respectively. Cells were incubated at 37°C in DMEM containing 0.3% bovine albumin (MP Biomedicals) and 1 µg/ml of tolylsulfonyl

phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). Supernatants were collected at selected time points postinfection (p.i.), and viral titers on MDCK cells were determined in a standard plaque assay.

Evaluation of SAα2,6 and SAα2,3 on human DC surfaces. Dendritic cells were treated with *Clostridium perfringens* neuraminidase (Roche) or heat-inactivated neuraminidase (incubated for 20 min at 95°C) for 2 h at 37°C. Then, the cells were incubated with 20 μg/ml of the biotinylated lectins *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAAI) (Vector Laboratories) for 15 min at 33°C, subsequently washed with PBS-1% BSA, and incubated with streptavidin-FITC for 1 h at RT. Data were acquired by flow cytometry using a FACScan and analyzed with FlowJo software. Neuraminidase-treated cells were used as a negative control for the presence of sialic acid. For fluorescence microscopy analysis, DAPI (4′,6-diamidino-2-phenylindole; 1 μg/ml) was added to the cells during the incubation with streptavidin-FITC, and cells where fixed and mounted for analysis in a fluorescence microscope (Zeiss Axioplan 2).

Infections of primary human cells with the recombinant influenza viruses. The primary human DCs were infected with recombinant influenza viruses at an MOI of 1, using serum-free DC medium for 45 min at 37°C as previously described (12). Then, DCs were plated in complete DC medium (10% FCS) at 1×10^6 cells/ml and incubated for 4 h at 37°C. Subsequently, cells were recovered by centrifugation for 10 min at $400 \times g$, and cell pellets were lysed for RNA isolation, while the supernatants were tested for cytokine production by multiplex ELISA.

Macrophages were infected at an MOI of 2 with serum-free DC medium for 45 min at 37°C, and then complete DC medium was added and incubated for 4 h at 37°C. Supernatants where recovered for multiplex ELISA cytokine analysis, and RNA from the cells was isolated for quantitative reverse transcription-PCR (qRT-PCR) analysis.

Human tracheobronchial epithelial (HTBE) cells, cultured in 12-mm Transwell filters, were washed 10 times with BEGM prior to infection in order to remove mucins. For cytokine induction evaluation, cells were infected at an MOI of 2 in 100 μl BEGM inoculum for 1 h at 37°C. Then, cells were washed once and a 1:1 BGEM-DMEM mixture was added to the apical and basal compartments of the wells. At 4 h, 24 h, and 48 h p.i., medium from apical and basal chambers was harvested and stored at −20°C for subsequent cytokine evaluation, and cells were lysed and kept at −80°C for qRT-PCR analysis. For replication assessment, at the desired time points, 100 μl of PBS was added over the infected cells, which were cultured at an air-liquid interphase. After 30 min of incubation at 37°C, PBS was removed and the titer of virus present in the wash was determined by plaque assay.

RNA isolation. RNA from human dendritic cells was extracted from 5×10^5 cells using an Absolutely RNA microprep kit (Stratagene). The concentration was evaluated in a spectrophotometer at 260 nm, and 500 ng of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions.

qRT-PCR. Evaluation of the expression of cytokines from different cell types was carried out using iQ SYBR green Supermix (Bio-Rad) according to the manufacturer's instructions. The PCR temperature profile was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. The mRNA level of each sample for each gene was normalized to α-tubulin and rps11 expression. The primers used for detection of the M protein from A/PR/8/34 influenza virus fragment RNA were 5′-TCAGGCCCCCTCAAAGCCGA-3′ (forward) and 5′-GGGCACGGTGAGCGTGAACA-3′ (reverse). For IFN-β quantification, we used 5′-GTCAGAGTGGAAATCCTAAG-3′ (forward) and (5′-ACAGCATCT GCTGGTTGAAG-3′ (reverse), for tumor necrosis factor alpha (TNF-α), 5′-A GTGAAGTGCTGGCAACCAC-3′ (forward) and 5′-GAGGAAGGCCTAAG GTCCAC-3′ (reverse), for RANTES, 5′-TTGCCAGGGCTCTGTGACCA-3′ (forward) and 5′-AAGCTCCTGTGAGGGGTTGA-3′ (reverse), and for RIG-I, 5′-AAAGCCTTGGCATGTTACAC-3′ (forward) and 5′-GGCTTGGGATGT GGTCTACT-3′ (reverse). For amplification of α-tubulin, 5′-GCCTGGACCAC AAGTTTGAC-3′ (forward) and (5′-TGAAATTCTGGGAGCATGAC-3′ (reverse) were used, and in the case of rps11, 5′-GCCGAGACTATCTGCACTA C-3′ (forward) and 5′-ATGTCCAGCCTCAGAACTTC-3′ (reverse) primers were used. All the reactions were performed in duplicate. The primer efficiencies for qRT-PCR were evaluated and in all cases were confirmed to be approximately 100%. CXF Manager software (Bio-Rad) was used to analyze the relative mRNA expression levels by the change in threshold cycle (ΔC_T) method using the two housekeeping genes for α-tubulin and rps11 to normalize the results. Thus, the nonnormalized relative expression of each gene was obtained with the formula $2^{[C_T(\min) - C_T(\text{sample})]}$, where C_T(min) is the average C_T for the sample with the minimal average C_T. The normalized relative expression was obtained by dividing the previous formula by the geometric mean of the relative expression of the α-tubulin and rps11 housekeeping genes.

Multiplex ELISA. Quantification of IP-10, TNF-α, MIP-1β, and interleukin 6 (IL-6) release in DCs, macrophages, and HTBE supernatants after infection was performed using the Milliplex multianalyte profiling human cytokine/chemokine kit (Millipore) according to the manufacturer's instructions. Data were analyzed using the Multiplex data analysis software.

Statistics. Results are presented as average values of results from replicates ± standard deviations (SD). Average values were compared by the unpaired Student t test. Experiments performed with DCs and macrophages were performed at least with three donors, and the results of two representative experiments are shown in every case.

RESULTS

The mutations Q226L and G228S change the receptor specificity of the H5N1 A/Vietnam/1203/2004 HA. In order to study the effect of receptor binding specificity in human primary immune cells, we used recombinant viruses that were identical in sequence except in the receptor binding domain of the HA. Viruses encoding HA with the WT sequence (Viet WT), HA with the mutations Q226L and G228S (Viet Mut), NA from the highly pathogenic A/Vietnam/1203/2004 H5N1 virus, and the six internal proteins for the A/PR/8/34 virus were generated by reverse genetics. The polybasic cleavage site of the HA of these viruses was mutated to reduce its virulence (39).

We first assessed the receptor specificities of the viruses Viet WT and Viet Mut using a flow cytometry-based assay. Briefly, MDCK cells were infected with these two viruses and also with another virus bearing the HA and NA genes from the seasonal influenza A/Brisbane/59/2007 H1N1 virus as a control for SAα2,6 binding specificity (Fig. 1A and B). Incubation with the 3′ SLN-PAA and 6′ SLN-PAA glycans indicated that 99% of the cells infected with the Viet WT virus bound to SAα2,3 but showed no detectable binding to the 2,6-linked SA. However, those cells infected with Viet Mut virus, which has the mutations Q226L and G228S in the HA, showed a significant reduction in binding to 2,3-linked SA and a striking increase in the affinity for SAα2,6. Specifically, 92% of cells infected with the Viet Mut virus bound SAα2,6, levels similar to those seen with the HA from the seasonal H1N1 virus.

To confirm these results, we also performed a solid-phase binding assay as described in Materials and Methods. As shown in Fig. 1C, the binding assay using the 3′ SLN-PAA showed that the Viet WT virus had a strong affinity for 2,3-linked SA, but a reduction in the case of the Viet Mut virus was observed. On the other hand, Viet WT showed low absorbance values for the 6′ SLN-PAA, but consistently with the flow cytometry results, the virus with the mutations Q226L and G228S in the HA showed high levels of binding to the 2,6-linked SA. Taken together, results from Fig. 1 show that the introduction of the Q226L and G228S mutations into the HA of Viet WT changed the receptor preference for that HA from α2,3- to α2,6-linked SA.

SAα2,6-binding Viet Mut replicates to higher titers in cell lines and in primary epithelial human cells than Viet WT. In order to characterize the viruses with different receptor specificities, we determined growth curves in the permissive MDCK and A549 cell lines (Fig. 2A and B). The Viet Mut virus, with preferential specificity for SAα2,6, replicated in MDCK cells similarly to Viet WT, showing a difference, an ~10-fold higher titer, at 24 h postinfection (p.i.), consistent with the presence of both SAα2,6 and SAα2,3 linkages on those cells (17). A549 cells also supported increased replication of Viet Mut com-
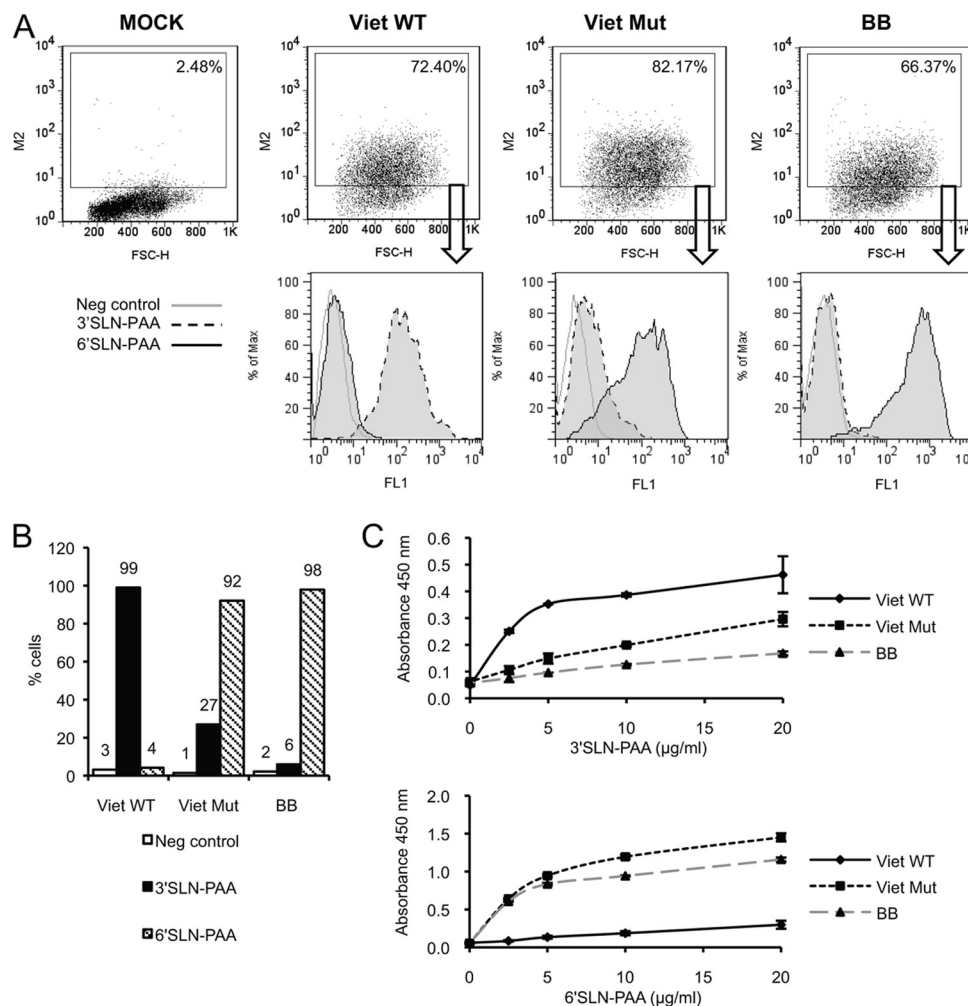
FIG. 1. Evaluation of the receptor binding specificities of the recombinant viruses Viet WT (HALo and NA from A/Vietnam/1203/2004), Viet Mut (HALo Q226L G228S and NA from A/Vietnam/1203/2004), and BB (HA and NA from A/Brisbane/59/07). (A) Flow cytometry binding assay; (B) percentages of infected cells binding to SAα2,3 or SAα2,6 by flow cytometry; (C) solid-phase binding assay. M2, M2 antibody; FSC-H, forward scatter height; 1K, 1,000; FL1, first fluorescence.

pared to that of Viet WT at 24 h p.i., although in this case, the differences were observed also at later time points, showing 10-fold differences for the maximum titers.

We also characterized the replication of these two viruses in human tracheobronchial epithelial cells (HTBE). Similarly to what was seen in A549 cells, the virus with affinity for SAα2,6 grew more efficiently in HTBE cells than the virus with the H5 WT HA using either a low or a higher starting MOI (0.1 or 2 PFU/cell), as shown in Fig. 2C and D. Infecting with an MOI of 0.1, we observed that the virus Viet Mut released about 100 times more virus than Viet WT at 72 h p.i.

Therefore, the virus with human-virus-like receptor specificity showed increased fitness in epithelial cell lines, as well as in differentiated primary human respiratory cells, compared to the virus with avian influenza virus receptor specificity.

Human DCs contain both SAα2,3 and SAα2,6 on their surfaces. To assess the presence or absence of SAα2,3 and SAα2,6 on the surfaces of human DCs and thereby predict their susceptibility to influenza viruses with distinct receptor specificities, we incubated monocyte-derived DCs with the biotinylated

plant lectins SNA and MAAI, which have affinity for α2,6- and α2,3-linked SA, respectively, and then with streptavidin linked to FITC (see Materials and Methods). To remove the sialic acids present on the surfaces of DCs as a negative control, we added neuraminidase from *Clostridium perfringens*, whereas heat-inactivated neuraminidase was added as a control in the rest of the samples. Flow cytometry analysis revealed that virtually all DCs presented both α2,6- and α2,3-linked SA on the cell surface (Fig. 3A), and these results were confirmed by fluorescence microscopy (Fig. 3B). This experimental system does not allow us to compare the levels of expression of α2,3- and α2,6-linked SA on DCs, since the lectins used may have differing affinities for their ligands. Nevertheless, these data indicate that human DCs can potentially be infected by influenza viruses with different receptor binding specificities (both α2,3- and α2,6-linked SA).

Viet WT virus, with a higher affinity for α2,3-linked sialic acids, induces a strong activation of human dendritic cells. We next studied cytokine and chemokine expression profiles in primary human DCs after infection with the recombinant vi-
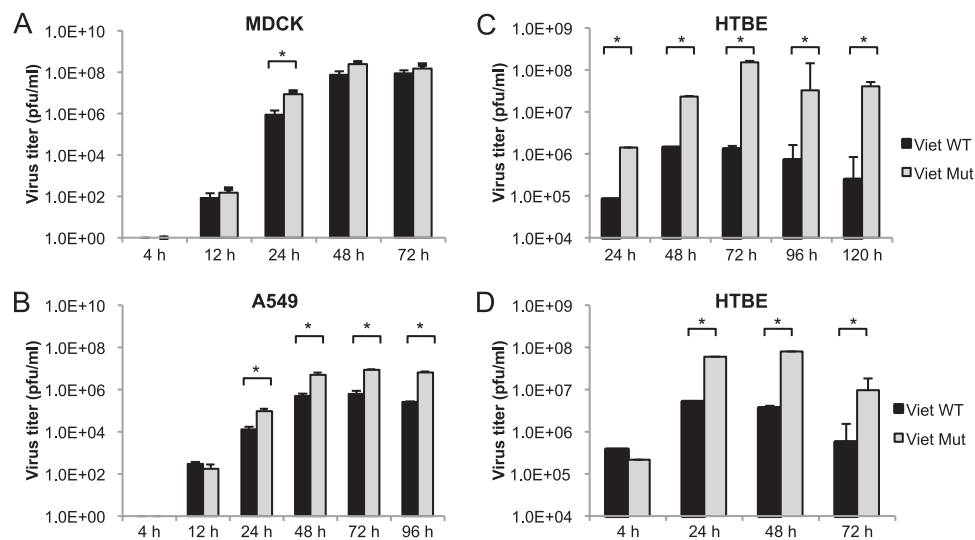
FIG. 2. Characterization of the recombinant Viet WT and Viet Mut viruses. MDCK cells (A) and A549 cells (B) were infected (MOIs, 0.001 and 0.1, respectively) with the Viet WT HA- and Viet Mut (G226S Q228L) HA-expressing viruses. HTBE cells were infected with the same viruses at an MOI of 0.1 (C) or 2 (D). At the indicated time points after infection, virus titers in the supernatants were determined by plaque assay on MDCK cells. Average titers ± SD are indicated. Statistically significant differences between the results of Viet WT and Viet Mut infections are represented by asterisks (∗, P value ≤ 0.05).

ruses Viet WT and Viet Mut. First, replication levels were evaluated by quantifying the levels of influenza virus M gene RNA by qRT-PCR (as described in Materials and Methods). At 4 h p.i., both viruses showed similar levels of replication in these cells. However, at 20 h p.i., the virus with specificity for SAα2,6 (Viet Mut) showed consistently higher levels of replication than Viet WT (Fig. 4A) in DCs obtained from two different donors. These results suggest that although human
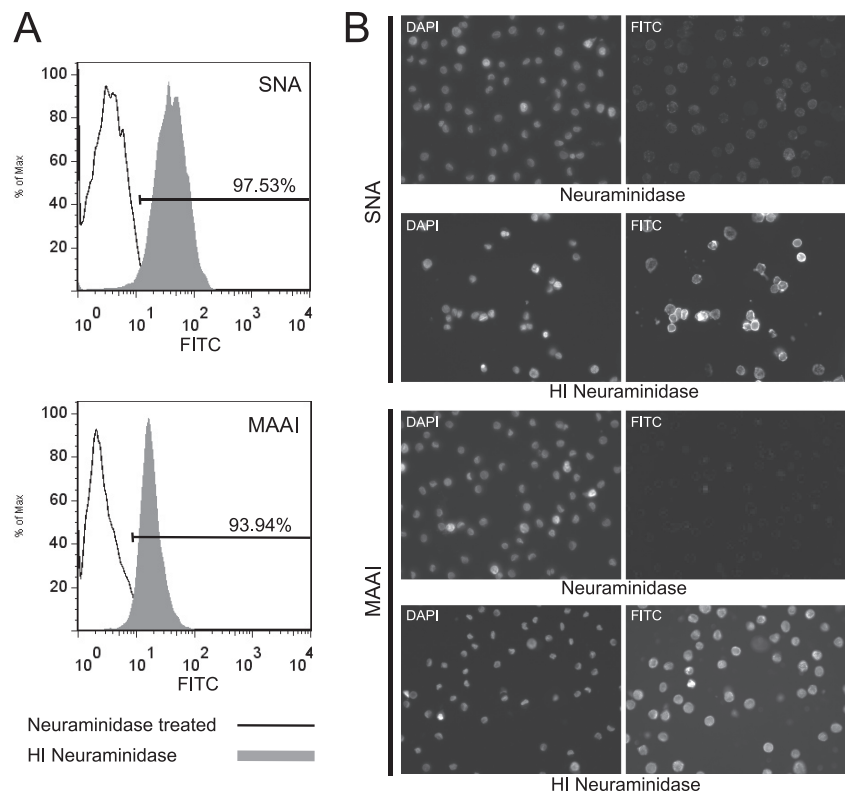

FIG. 3. Evaluation of SAα2,3 and SAα2,6 in human DCs. Cells were treated with neuraminidase from *Clostridium perfringens* that was either active or heat inactivated (HI), incubated with biotinylated SNA or MAAI, and analyzed by flow cytometry (A) and fluorescence microscopy (B).
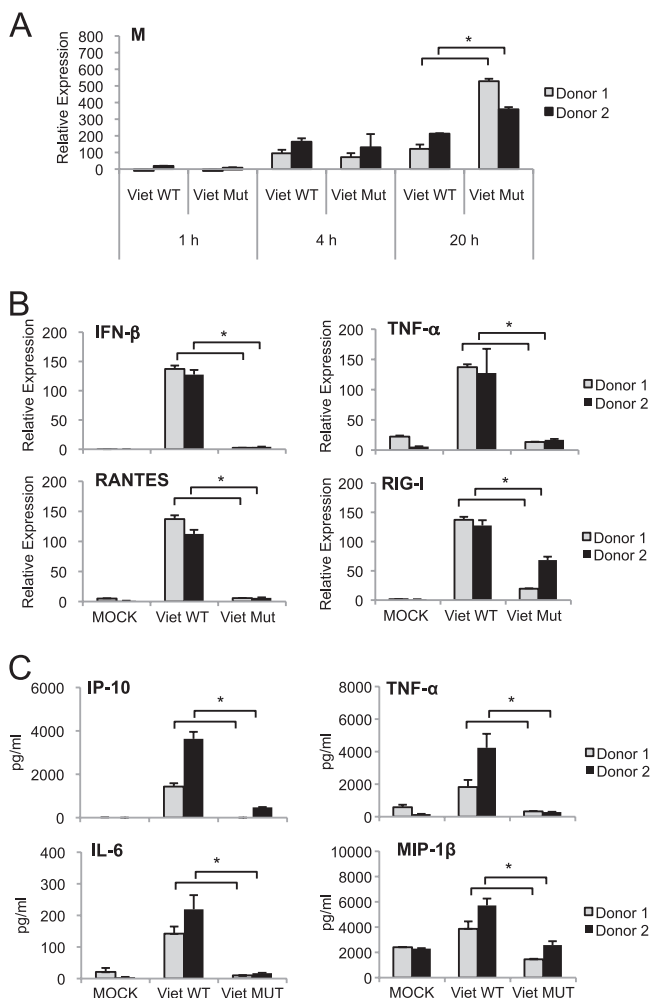
FIG. 4. Evaluation of the expression of genes associated with the proinflammatory response in human DCs induced by the viruses with SAα2,3 (Viet WT) and SAα2,6 (Viet Mut) receptor binding specificity. (A) Analysis of the replication of Viet WT and Viet Mut; (B) comparison of the levels of expression of the indicated genes by qRT-PCR at 4 h p.i.; (C) evaluation of cytokines and chemokines released to supernatants at 4 h p.i. Results from two representative donors are shown. Average results from replicates ± SD are indicated. Statistically significant differences between the results of Viet WT and Viet Mut infections are represented by asterisks (∗, P value ≤ 0.05).

DCs express both α2,3- and α2,6-linked SA, viruses with affinity for the α2,6-linked SA replicate more efficiently in those cells.

Since we are interested in early events in the innate immune recognition of influenza viruses by human DCs, and to eliminate possible differences in DC activation by these two viruses due to differential replication in DCs, we evaluated the expression of genes associated with the proinflammatory response at 4 h p.i. By qRT-PCR, our data show (Fig. 4B) that Viet WT virus, with SAα2,3-preferential specificity, induced elevated levels of mRNA IFN-β and TNF-α in infected DCs, while those observed upon Viet Mut infection were comparable to the basal levels. These results were highly reproducible among different donors (data not shown). Also, the Viet WT virus induced significantly greater expression of RIG-I and RAN-

TES in infected DCs than did the Viet Mut virus (Fig. 4B). Additionally, we analyzed the concentration of several cytokines and chemokines secreted by the cells to the supernatants in the infected cell cultures by multiplex ELISA (see Material and Methods). As shown in Fig. 4C, DCs infected by the virus with SAα2,3 specificity released high levels of IP-10, indicative of IFN production, whereas in the case of DCs infected with Viet Mut, which had SAα2,6-preferential binding, very small amounts of this chemokine were detected. We obtained similar results when we evaluated the levels of the proinflammatory cytokines TNF-α and IL-6 and the chemokine MIP-1β, indicating that the virus with SAα2,3-preferential binding induced a stronger proinflammatory response in human DC than the virus with SAα2,6 binding specificity. To ensure that this differential response was not due to possible contaminants in the allantoic fluid or cell supernatant of the virus stocks, sucrose cushion-purified viruses (grown in MDCK cells or in eggs) were also tested, and similar results were obtained (data not shown). These results—based on a comparison of influenza viruses which differ by only 2 amino acids—indicate that preferential binding of HA to SAα2,3, but not to SAα2,6, induces a high proinflammatory response in human DCs at early times postinfection.

Differences in DC activation by SAα2,3- and SAα2,6-binding influenza viruses are independent of viral replication. The experiments described above suggested that the differences in activation were independent of viral replication, since we did not observe differences in replication at the time when the cytokine profile was analyzed and because the distinct activation pattern was observed at an early time point. To test this hypothesis, we performed the infections with UV-inactivated viruses. We first confirmed the lack of replication of the UV-inactivated preparations by qRT-PCR (Fig. 5A) and plaque assay and then studied the expression of proinflammatory genes. We observed that the UV-inactivated Viet WT virus was able to induce the expression of IFN-β and TNF-α in a way similar to that of the noninactivated viruses, but we did not observe an upregulation of these genes in the case of those cells infected with the virus Viet Mut (Fig. 5B). Analysis of RIG-I and RANTES expression revealed a similar result. As demonstrated in Fig. 5C, the analysis of supernatants by multiplex ELISA revealed an elevated production of the cytokines TNF-α and IL-6 and of the chemokines IP-10 and MIP-1β following infection with either UV-inactivated or live viruses with SAα2,3 specificity relative to that of noninfected cells and those infected with the SAα2,6-binding virus. Together, these data indicated that the high activation of DCs induced by viruses with α2,3-linked SA specificity is independent of replication.

Human primary macrophages produce higher levels of cytokines upon infection with virus possessing SAα2,3 receptor preference than with virus possessing SAα2,6 receptor preference. To elucidate whether the observed differential response was specific for DCs or extended to other human immune cell subtypes, we tested the responses induced by the Viet WT and Viet Mut viruses in human macrophages. We first assessed viral growth in this substrate using the M segment-based qRT-PCR assay. As in DCs, at 4 h p.i., the levels of replication were comparable between the two viruses, but at 20 h p.i. the virus with affinity for SAα2,6 showed a higher level of replication. In
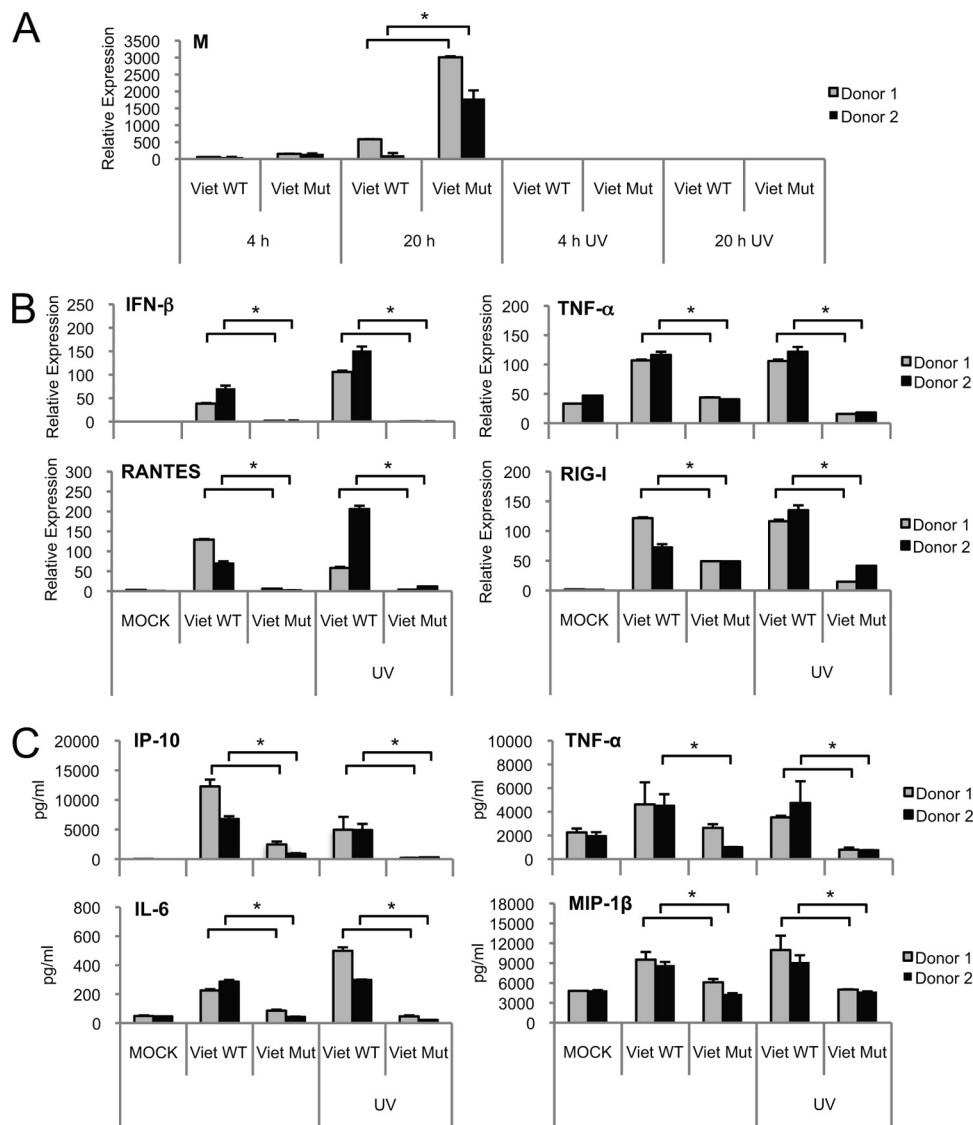
FIG. 5. Comparison of the cytokine expression profiles induced by noninactivated and UV-inactivated viruses with different receptor specificities in human DCs by qRT-PCR at 4 h p.i. (A) M expression in infected DCs; (B and C) expression of proinflammatory genes by qRT-PCR and multiplex ELISA, respectively. Results from two representative donors are shown. Average results from replicates ± SD are indicated. Statistically significant differences between the results of Viet WT and Viet Mut infections are represented by asterisks (∗, P value ≤ 0.05).

the case of this cell type, the pattern of cytokine and chemokine production as a consequence of infection was similar to that observed in DCs, showing a higher expression of IFN-β and TNF-α, as well as of other genes tested (Fig. 6), following infection with the virus possessing the SAα2,3-binding HA than following infection with the virus with the SAα2,6-binding HA. These results indicate that the phenotype observed for these viruses is not unique to DCs. Also, they show that macrophages, which are one of the main cytokine producers of the innate immune system, show a higher activation phenotype following infection by an SAα2,3-binding virus than following infection by an SAα2,6-binding virus.

Differential expression of proinflammatory responses in human respiratory epithelial cells by SAα2,3- and SAα2,6-binding influenza viruses. The respiratory epithelium and mucosa is the first tissue with which influenza viruses interact, and

therefore the innate immune response induced in cells of this tissue is crucial for immune cell recruitment and, eventually, virus clearance (51). In order to elucidate if the viruses with differing receptor specificities also induced different innate immune responses in respiratory human cells, we tested the recombinant viruses with different receptor specificities in HTBE cells. Consistent with the release of viral particles by virus titration as described above, the analysis by qRT-PCR showed higher levels of mRNA of the M segment in cells after infection with the virus Viet Mut than after infection with the virus Viet WT (Fig. 7A). As observed in Fig. 7B, the virus Viet WT showed higher levels of expression of IFN-β and the interferon-inducible chemokine IP-10 at 24 and 48 h p.i. than Viet Mut, as evaluated by qRT-PCR. Also, TNF-α and IL-6 mRNA levels were notably high at 48 h p.i. in cells infected with the virus with SAα2,3 specificity (16.8- and 11.5-fold over those in

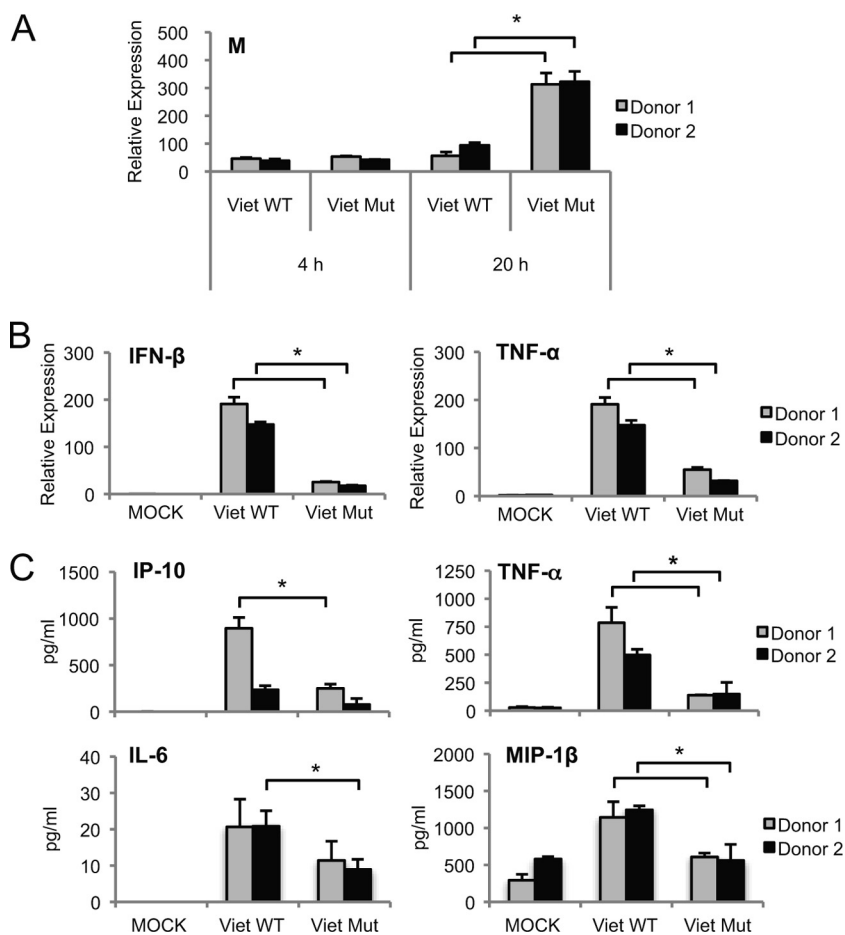FIG. 6. Comparison of the cytokine expression profiles induced by the viruses with SAα2,3 (Viet WT) and SAα2,6 (Viet Mut) receptor binding specificities in human macrophages at 4 h p.i. (A) M expression in infected macrophages; (B and C) expression of proinflammatory genes by qRT-PCR and multiplex ELISA, respectively. Results from two representative donors are shown. Average results from replicates ± SD are indicated. Statistically significant differences between the results of Viet WT and Viet Mut infections are represented by asterisks (∗, P value ≤ 0.05).

mock-infected cells), while those infected by SAα2,6-specific viruses showed levels only slightly higher than those in the uninfected cells (3.1 and 4.6 times over those in mock-infected cells). The expression of the chemokines IL-8 and RANTES presented similar patterns, showing the greatest differences between the cells infected with the SAα2,3- and SAα2,6-binding viruses after 48 h of infection. The release of IP-10, TNF-α, IL-6, and MIP-1β was also analyzed in the supernatants in the apical compartment of the HTBE cultures (Fig. 7C). Production of IP-10 was detected at 48 h p.i. in cultures infected with both the Viet WT and Viet Mut viruses, being about 8.5-fold higher than those observed in Viet WT than in Viet Mut. Elevated production of TNF-α, IL-6, and MIP-1β was detected in supernatants from cultures infected with Viet WT (2,400.5 ± 909.6 pg/ml, 1,074.7 ± 272.2 pg/ml, and 105.3 ± 12.5 pg/ml at 48 h p.i., respectively), while levels observed in Viet Mut-infected HTBE cells were similar to those observed in mock-infected cultures. Comparable results were obtained when we evaluated the production of IP-10, TNF-α, IL-6, and MIP-1β in the lower chamber of the HTBE cultures (data not shown). Therefore, the virus with α2,3-linked SA specificity induced a stronger inflammatory response in primary respira-

tory cells than that with α2,6-linked SA specificity. It is important to note that the expression of proinflammatory molecules is delayed in epithelial cells compared to that in immune cells, since, unlike with DCs and macrophages, we were not able to detect differences at 4 h p.i. between uninfected and infected cells in the HTBE cultures.

DISCUSSION

H5N1 HPAIV have expanded throughout Asia and some parts of Africa and Europe in the last decade. This expansion is associated with widespread death in poultry (which has had an important economic impact on the poultry farming industry) and has resulted in more than 500 human infections to date. The high pathogenicity of H5N1 influenza viruses and their ability to transmit from birds to humans has become a major concern worldwide, and although the virus has not yet acquired the capacity for sustained human-to-human transmission, it continues to undergo genetic changes that may result in the acquisition of this capacity (1, 14, 41, 53), making these viruses a potential pandemic threat. Although the factors that determine efficient human-to-human transmission are not
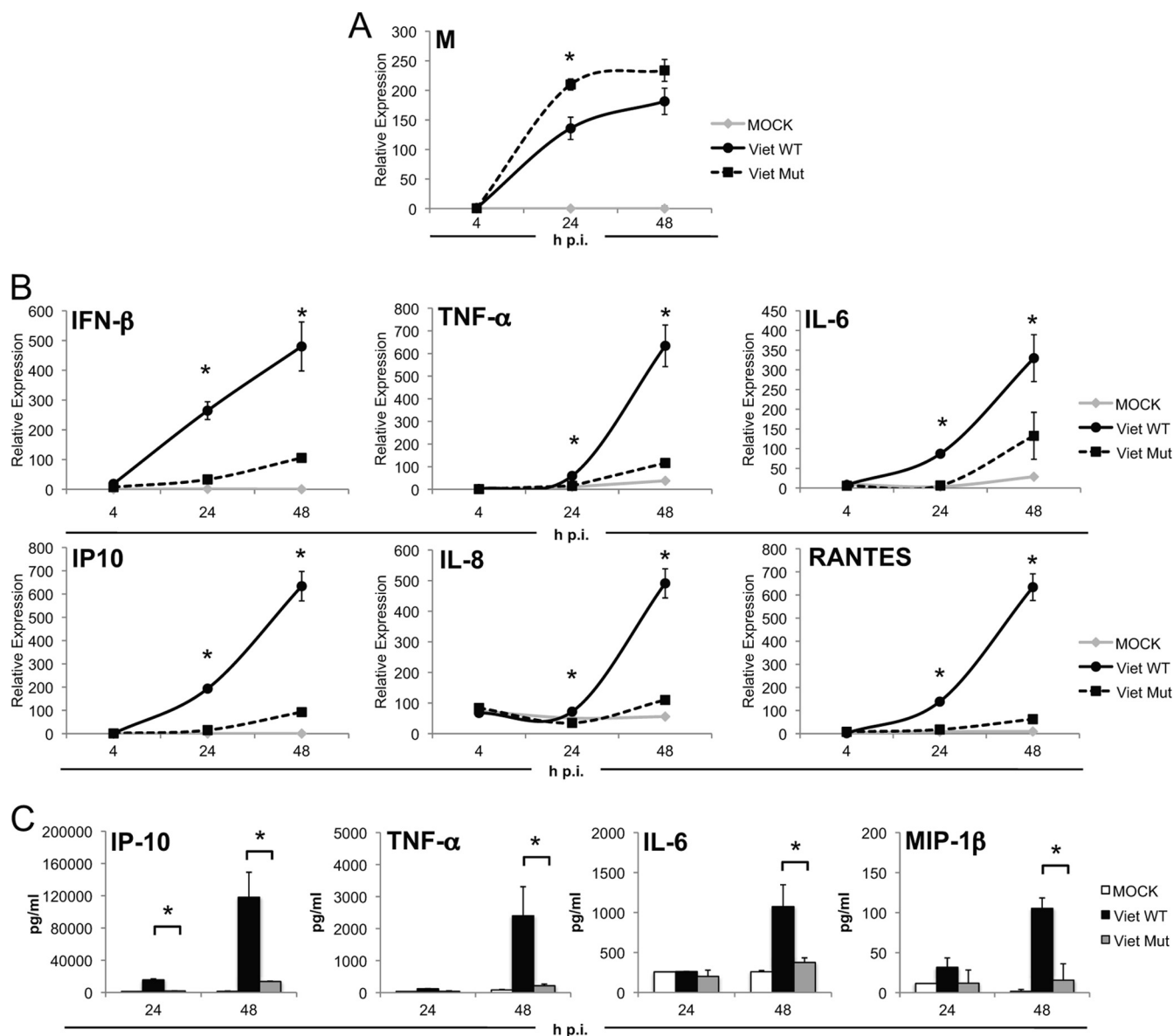
FIG. 7. Analysis of the production of cytokines and chemokines induced by the viruses with SAα2,3 (Viet WT) and SAα2,6 (Viet Mut) receptor binding specificity in HTBE cells. (A) Evaluation of the M expression by qRT-PCR; (B) comparison of the levels of expression of the indicated genes by qRT-PCR at the indicated times p.i.; (C) evaluation of cytokines and chemokines released to the supernatants at the indicated times p.i. Average results from triplicates ± SD are indicated. Statistically significant differences between results of Viet WT and Viet Mut infections are represented by asterisks (∗, P value ≤ 0.05).

completely understood (29), it is thought that a change of receptor specificity from α2,3- to α2,6-linked SA of the viral HA is essential for facilitating transmission between humans (47). As a consequence, several studies have identified specific mutations in the influenza virus HA that can change HA's receptor specificity from SAα2,3 to SAα2,6, which may confer on avian influenza viruses the ability to be transmitted among humans (8, 41–43, 53). Here, we analyzed the HA receptor specificity of the A/Vietnam/1203/2004 H5N1 HPAIV and the effect of the amino acid changes Q226L and G228S in the HA of this isolate, using two different methods (a fluorescence-activated cell sorting [FACS]-based assay and a solid-phase binding assay), confirming the previously reported observation

that these mutations changed the preferential receptor specificity of the H5N1 influenza virus from SAα2,3 to SAα2,6 (8, 41, 43). The work by Stevens et al. in 2006 (43) first described the effect of these mutations in the recombinant HA from the A/Vietnam/1203/2004 virus by using a glycan array to test the binding of the protein to different glycans. In that work, although they did not see a dramatic shift, they observed a considerable reduction of binding to SAα2,3 and significant binding to SAα2,6 glycans. Later, another report from the same authors showed similar experiments performed with the whole virus, showing a more remarkable shift in preferential binding than when the recombinant purified protein was used (41). Therefore, in those previous reports, the A/Vietnam/

1203/2004 HA with the mutations Q226L and G228S showed some levels of binding to SAα2,3 glycans. Here, using two different assays, we observed a switch in the preferential binding after introducing the changes Q226L and G228S in the HA of this H5N1 virus. However, in concordance with those previously reported data, low remaining levels of binding to SAα2,3 by the Viet Mut virus were also observed.

Given that the severity of human infections by HPAIV is believed to be associated with the induction of a strong inflammatory response in the host (11, 32, 46), we hypothesized that the receptors to which the avian or human influenza viruses bind and how they bind to the cell surface receptors may have an effect on the way that they are "sensed" by the immune cells in the lung and therefore induce a different immune response, contributing to the hyperinduction of proinflammatory cytokines observed in humans infected with avian viruses. Differential expression of proinflammatory genes by seasonal human and highly pathogenic avian viruses has also been observed in human primary dendritic cells (34, 45) and macrophages (7, 16, 23, 24, 55). Due to the important role of the DCs in initiating innate immune responses as a consequence of the recognition of PAMPs and their ability to produce proinflammatory cytokines following activation, we first investigated the effect of the receptor specificity of the influenza viruses in human primary DCs. As shown in Results, we observed that the virus with α2,3-linked SA receptor specificity induced a higher expression of proinflammatory genes by DCs and macrophages (Fig. 4 and 6), and this effect was also observed using UV-inactivated viruses (Fig. 5), which indicates that the recognition of the virus that binds SAα2,3 may occur differently from that of the virus with SAα2,6 specificity. The response that is induced is independent of the replication of the virus. Consistently with this, Miller and Anders (28) observed that inactivated influenza viruses induced type I IFN in murine splenocytes and that the interaction of the virus with the sialylated receptors on IFN-producing cells was required. Sialic acids are structural determinants of the cell surface, and there is increasing evidence of their importance in immune system modulation (3, 10, 35, 38, 44, 50), although there are still numerous unknown aspects in the interaction of the influenza virus with their receptors. As shown above (Fig. 3), human DCs contained both α2,6- and α2,3-linked SA on their surfaces, and therefore they are potentially susceptible to infection with both avian and human viruses. Nevertheless, little information has been reported to date regarding sialylation levels of surface glycoproteins in either immune or epithelial cells.

The respiratory tract is the first barrier of defense against influenza viruses and other respiratory pathogens, conferring mechanical protection through cilia and mucus but also releasing proinflammatory cytokines and chemokines that stimulate the recruitment of immune cells. In this work, we show that the change of an avian virus to have a more human virus-like receptor specificity resulted in an increased ability to infect and replicate in HTBE cells, which resemble the epithelia of the human respiratory tract. Similarly, Matrosovich et al. (26) tested in differentiated HTBE cells the pandemic human A/Hong Kong/1/1968 (H3N2) virus, which presents SAα2,6 receptor specificity, and a mutant virus with the changes L226Q and S228G in the HA, which presents SAα2,3 receptor specificity. Consistent with our data, they observed a better infection and replication capacity for

the viruses with SAα2,6 receptor binding receptor specificity. Comparisons of the innate immune responses elicited by human and avian influenza viruses in human respiratory epithelial cells showing that avian influenza viruses induce a stronger inflammatory response than human influenza viruses have been reported elsewhere (5, 15, 52). Those studies used differing gene constellations, so that it is not possible to attribute the observed effects to a particular genetic factor. The work reported here is focused on the contribution of receptor binding specificity to the initiation of innate immune responses in human cells. Therefore, we used viruses that were different only in the receptor binding site of the HA and shared all the other genes. Thus, based on our data, we can attribute the differences in cytokine activation by the viruses tested to their different receptor specificities. Another recent report showed a higher induction of type I IFN in the epithelial cell line A549 after infection with influenza viruses bearing the HA and NA from two avian H5N1 viruses (A/Vietnam/1203/2004 and A/Hong Kong/213/2003) than viruses with the HA and NA from the H1N1 human influenza A/New Caledonia/20/1999 virus (6). In that work, the recombinant viruses constructed shared the six internal genes from the cold-adapted virus A/Ann Arbor/6/1960, which indicates that the differences in type I IFN induction are mediated by the HA and NA. Although we show that both immune and epithelial human primary cells present higher proinflammatory cytokines after infection with viruses that bind SAα2,3 than with viruses that bind SAα2,6, it is important to point out that the detection of cytokines was delayed in the epithelial cells, since at 4 h p.i., no differences from the mock-infected cells were observed.

Interestingly, high production of the chemokines IP-10, IL-8, and RANTES, which are involved in immune cell recruitment to infected tissues, were detected after infection with the virus with SAα2,3 receptor specificity in HTBE cells. Indeed, induction of high levels of IP-10 have been observed in human lungs (46) and in the sera of humans (32) infected by H5N1 virus. These data suggest that infection of lung epithelial cells by SAα2,3-binding viruses may result in higher recruitment of proinflammatory cells to the site of infection.

Taken together, our findings provide evidence to support the hypothesis that the strong host inflammatory responses induced in humans by H5N1 HPAIV could result from their SAα2,3 receptor specificity. Interestingly, our data strongly suggests the existence of two non-mutually exclusive scenarios. One is that the receptors on human DCs and macrophages that sense SAα2,3-binding viruses are distinct from those that sense SAα2,6-binding viruses, resulting in a more rapid and enhanced proinflammatory response in the lungs of infected patients after binding by SAα2,3-preferential viruses. Alternatively, differential recognition of SAα2,3- and SAα2,6-binding viruses by the same receptor on human immune cells could result in distinct signaling cascades of activation in those cells. Further studies related to the recognition of influenza viruses by immune cells would help to clarify and help us understand the mechanisms involved in the induction of hypercytokinemia by highly pathogenic influenza viruses in humans.

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